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Review Article

A Systematic Guide on HPLC Troubleshooting

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ABSTRACT

In compared to prior approaches, HPLC provides far higher resolution, more accurate quantitative results, and quicker analysis durations. As a result, HPLC has developed into a vital instrument in many analytical labs. Troubleshooting is a type of problem solving that is frequently used to fix failing processes or goods. Actually, the term "HPLC" is used to describe a variety of separation methods that employ a liquid mobile phase, or eluent. Understanding the fundamental principles underlying the operation of the instrument and the separation is necessary for troubleshooting HPLC equipment and separations. Component faults (pump, degasser, injector, detector, data system, column) and improper mobile phase or sample preparation are the main causes of HPLC issues. For best results, always prepare food in clean glasses. Cleaning the HPLC with the proper solvent is the primary solution to HPLC issues. It is advised to do a fast visual inspection of the instrument and column if any issue arises. Try to follow the same procedure if a monograph or other protocol is supplied. Troubleshooting is a type of issue resolution that is frequently used to fix failing processes or goods. Alternatively, we advise you to read the complete article to learn some tips that will enable you to prevent issues in the future. We hope that this article will help you detect issues and understand their underlying causes so that you can stop or lessen their occurrence in the future. The most crucial aspect of troubleshooting is the care of the colon and HPLC. We attempted to address all significant troubleshooting issues in this post.

INTRODUCTION

Safe laboratory procedures must be followed before to beginning any troubleshooting, whether it concerns instruments or columns. Any solvents used should have a material safety data sheet (MSDS) that is easily accessible and that details their chemical and physical characteristics. Troubleshooting is a type of problem-solving that is frequently used to fix faulty items or procedures.

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It entails a logical, methodical investigation for a problem's root cause so that it may be fixed and the product or process can resume normal operation. Complex systems need troubleshooting to build and maintain since there are several potential sources for a problem's symptoms. Engineering, system administration, electronics, automobile repair, and diagnostic medicine are just a few of the industries that require troubleshooting. Identification of the system's malfunction(s) or symptoms is necessary for troubleshooting. The next step is to generate potential reasons for the symptoms using experience [1-2]. Being unable to use an HPLC might be quite annoying. For instance, you're trying to do some analysis, but you can't proceed with your investigation until you identify the cause of your instruments' malfunction.

Occasionally, HPLC columns will experience a few common issues. You may avoid hours of frustration by understanding what they are and how to remedy them. Continue reading to learn about some of these issues and solutions. This article is only basic in nature. The HPLC system's manufacturer should be consulted, as we would advise. Cleaning HPLC with the proper solvent is the main step in solving HPLC issues [10].

Types of HPLC

Depending on the substrate used i.e. stationary phase used, the HPLC is divided into following types [11]

- Normal Phase HPLC: Polarity is used in this procedure to separate the components. Hexane, chloroform, and diethyl ether are employed as the non-polar stationary phase while silica serves as the primary polar stationary phase. On a column, the polar samples are kept.
- HPLC in reverse phase: This is the opposite of HPLC in normal phase. The stationary phase is hydrophobic or non-polar whereas the mobile phase is polar. The non-polar character will be kept more the more of it there is.
- With size-exclusion HPLC, precisely regulated substrate molecules will be incorporated into the column. The separation of components will take place based on the variation in molecular sizes.
- Ion-exchange HPLC: The stationary phase has an ionized surface that is opposite the charge of the sample. Aqueous buffer, which is the mobile phase utilized, will regulate the pH and ionic strength [11].

In order to better understand how different molecules function within complicated mixtures of molecules found in chemical and biological systems, high performance liquid chromatography, or HPLC, is utilized. The first HPLC techniques for the analysis of bulk medicinal compounds came in the year 1980. (United States Pharmacopoeia, 1980). [1, 29]

Gravimetric analysis, photometry, colorimetry (UV, visible detection), titrimetry (acid-base detection), and other techniques were the only ones accessible for analysis prior to the discovery of chromatography. Even the requirements for analysis in research were straightforward; for example, it wasn't necessary to analyze complicated or comparable substances. (i.e., molecules with the same chemical and physical properties). However, as research developed, it became necessary to evaluate every molecule in a sample in order to better identify the issue (in the clinic), contaminants, as well as shortcomings in both industry and research. Due to the increasing physical and chemical similarity in molecules of a sample, such as phytoconstituents, amino acids, and neurotransmitters, etc., this was not achievable with a single technique like photometric, titrimetric, etc. [15, 16]. Based on their affinity for the molecules in the column, the components in the sample are separated. The separated chemicals in the sample then go through the detectors. The

HPLC process uses user software for data analysis. [12].

Actually, the term "HPLC" is used to describe a variety of separation methods that employ a liquid mobile phase, or eluent. Understanding the fundamental principles behind the operation of the instrument and the separation is necessary for troubleshooting HPLC equipment and separations. This practical method is intended to be used as both a troubleshooting manual and a learning aid for HPLC [3].

Visual Inspection:

It is advised to do a rapid visual inspection of the instrument and column when a problem arises. This will detect leakage, disconnected or lost tubing, modifications to instrument settings, etc. [4, 9].

No Peaks:

Single or several missing peaks are typically caused by injecting the incorrect sample or by the sample deteriorating. However, a loss of resolution brought on by inconsistent column/solvent behavior is as probable. No peaks or very tiny peaks may appear on HPLC results due to a variety of causes. The peaks in a normal reading should be broad, thin, and may vary in height. Make sure your detector is turned on before checking all the electrical connections and cables. If you experience small peaks or no peaks at all, this could indicate that your detector lamp is off, that you lack mobile phase flow, that your sample is missing or deteriorating, or that there is a problem with your detector, integrator, or injector valve. Verify that the auto sampler vials are full of liquid and that the sample is free of air bubbles, then verify the system using a fresh standard solution. If it doesn't work, look at the state of the auto zero and attenuation or gain settings. Additionally, look for loose or damaged wires between the detector and the integrator or recorder, a lack of mobile phase flow, and an incorrect or degraded sample. Too-high detector or recorder settings [5].

No Flow

You could not have flow in your HPLC column if it is producing absolutely no peaks. This can indicate that your pump isn't working or that the flow is being impeded in some way. Additionally, the pump head may be airtight or have a leak. If the pump isn't running, turn it on and check the mobile phase levels in the reservoir and system flow. Make that the mobile phase is appropriately degassed, that the components are miscible, and that there are no blockages or air locks in the sample loop. Check for Pump off as well. Incomplete or impeded flow, any leaks, or air trapped in the pump head. After that, inspect the pump for leaks or other problems and the system for loose fittings. If your guard column has tubing, disconnect it and check the flow. If the issue persists, prime the system, release any system check valves, and, if all else fails, flush the system with 100% methanol or isopropanol. Purge the pump at a high flow rate [6].

Detector leaks

It is usually advised to investigate variables like potential cause, Cell gasket failure, high backpressure prevention, gasket replacement, shattered cell window(s), cell window replacement(s), leaking fixtures Replace or tighten fittings, obstructed sewer line Replace the waste line. A flow cell is blocked [4–9]. Additionally, choosing the appropriate HPLC detector for a particular application is advised [13].

Pressure

There may be a leak or air rap somewhere in the system if HPLC pressure is lower than expected or absent. A broken check valve or a blocked or stopped mobile phase flow are other potential issues. If your system pressure is excessive, there may be an issue with the pump, injector, in-line filter, tubing, or you may have analytical or guard columns that are clogged.

Start by disconnecting the injector and detector for excessive pressure by removing the guard and

analytical columns from the system and replacing them with unions. Work to isolate the cause by operating the pump at a rate of 2 to 5 mL per minute while starting with the detector, moving on to the in-line filter, and finishing with the pump. If the analytical column appears to be the source of the issue, reverse and flush the column while it is detached from the detector, and if required, replace the analytical column or change the intake frit.

Start by inspecting the whole system for leaks, frayed fittings, broken pump seals, and damaged valves if there is low pressure. Run the same mobile phase check we described before and look for flow at the analytical and guard columns if everything appears to be in order. Reconnect everything and try pumping solvent at double the flow rate if that doesn't work. Additionally, look for any leaks or interruptions to the mobile phase flow. Pump head leaks or any air pockets at the column inlet end fitting.

The viscosity of the solvent employed, column variables, flow rate, and temperature are some of the factors that have an impact on system pressure. When there are high or low pressures compared to the usual, it is crucial to establish a reference point. The pressure produced in the system when everything is working properly should serve as this point of comparison.

There are three types of pressure issues: high, low, or variable pressure. They could start slowly and then pick in speed. Particles from the sample, clogged or broken tubing, or column packed bed collapse are possible causes of a sudden pressure surge. Particles in the sample can also cause gradual pressure increases, but they can also be produced by the instrument itself, such as seal degradation or septa debris. Additionally, you can check for pump, injector, in-line filter, or tubing problems. guard column or analytical column obstruction [9].

PEAK AREA PRECISION SYMPTOM

Inappropriate detector settings

1) High, low, or changing pressure are the three different forms of pressure problems. They could begin gently before picking in speed. A quick pressure rise may be caused by sample particles, blocked or damaged tubing, or column packed bed collapse. The steady buildup of pressure due to particles in the sample can also be caused by particles created by the instrument, such as seal wear or septa debris. You can also look for issues with the pump, injector, in-line filter, or tubing. Obstruction of the analytical or guard columns.
2) Potential causes include a rapid response time, excessive noise, and inaccurate integration at the trace level. Solution: Ensure that the reaction time (or time constant) parameters are appropriate. Set your response time such that it is typically 1/4 of the narrowest peak's peak width at half-height. For information, refer to the operating instructions.
3) Nebulizer temperature error (Thermo Scientific Dionex Corona Detectors ultra (RS)) Possible Cause: Verify the temperature setting on the nebulizer. Set the temperature to 30 °C if you're using a lot of THF or other halogenated solvents in the mobile phase. In order to regain responsiveness, it might be essential to switch off the nebulizer heater if the analyte is semi-volatile.
4) Potential Cause: Insufficient data points Solution: For repeatable peak integration, set the data collection rate to at least 20–30 data points [10].

Injection volume variation

1) Potential Cause: The auto sampler removes air from the vial Verify the injector needle's sampling height and sample filling height.
2) Potential Cause: degrading samples Use suitable storage conditions, such as a thermostated auto sampler, as a fix.

3) Air in the auto sampler fluidics might be the issue. Solution: Use the procedures outlined in the relevant operating instructions to flush out the auto sampler fluidics.

Peak Integration Settings

Possible explanation: Positional variations of integration delimiters Solution: Verify the settings for the software integration. For example, using assistance from Thermo Scientific's Dionex Chromeleon. It is best to help the Chromeleon 7 Cobra algorithm find the optimal settings for you. Use a fixed data rate instead of an automated data rate setting [10].

Variable Retention Times

As RT increases, the composition of the mobile phase changes, the flow rate drops, and the mobile phase bubbles. Reduce RT: Boost flow, Overload the column, Active group on stationary phase Temperature: Per 1° Celsius increase in temperature, retention decreases by 1% to 2%. Pump Flow Rate Issue (verify real volume/delivery time)

- Incorrect Column Type (C8 – less retention, vs C18 – more retention)
- A temperature issue (warmer – less retention, colder – more retention)
- Organic Content in Mobile Phase, % (more organic – less retention, less organic –more retention)

Additional variables that may impact retention time include:

Temperature, Ion Pairing, pH Control, and Solvent Composition Drifting Column contamination, stability, equilibration, stationary phase, retention, and any leakage. composition of the mobile phase changing. (Small changes can lead to large changes in retention times.) a pump with air buildup. (Retention times fluctuate at random intervals. Variations in column temperature and column overload. (Retention times usualy decrease as mass of solute injected on column exceeds column capacity.) Sample solvent and mobile phase are incompatible. Column issue. (Not a frequent reason for inconsistent retention. Retention times gradually shorten as a column matures. Changes in the composition of the mobile phase, column chemistry, flow issues, and valve failure [3-9]

Column Back Pressure

It may be influenced by factors such as but not restricted to Locate Pressure Issue, In-Line Filter, Guard Cartridge, and Buffer Precipitation

Baseline problems:

Both cyclic and noncyclic baseline abnormalities are possible. They may result from column pollution, solvent impurities, detector malfunctions, electrical interferences, or detector defects, among other things. Determine if the issue is with the fluid flow, detector, or electrical connections in order to pinpoint the cause of a baseline irregularity [14].

Non-cyclic noise – Detector electronics problems

The detector is the most frequent root of issues with electronic baseline noise. The ensuing chromatogram will typically contain false peaks and some signs of baseline drift if the detector is not given enough time to equilibrate before an injection is done.

Loss of Resolution

The mobile phase became polluted or degraded, changing selectivity and/or retention times. Blocked analytical column or guard. If a standard or sample is utilized that has expired, the resolution may fail. Correct column and temperature are other factors that affect resolution.

Abnormal peak shape:

A variety of peak shape issues might be considered abnormal: Peaks that are fronting or tailing, absent peaks, smaller-than-expected peaks, larger-than-expected peaks, all-or-none double peaks, shouldering peaks, flat-topped peaks, or negative peaks

Broad Peak

The most frequent causes of broad peaks are instrumentation or column mistakes. The column and guards should be looked into first since they are frequently the most important component of the system.

Split Peak

You could have some contamination on your guard or analytical column inlet if peaks begin to appear that dip down in the center, forming a M shape. Additionally, the column inlet may have an uneven void or a partially obstructed frit. Another possibility is that the solvent in your sample is incompatible with the mobile phase [4].

If you suspect contamination at an intake, turn the column around and flush it. The column may also be used in reverse flow direction by refilling the top with pellicle particles that have the same bonded phase functionality.

Adjust the sample in the mobile phase if you believe it is the cause of the issue. Split peaks result from different pHs in the sample and mobile phase. contamination of the analytical column inlet or the guard. obstructed in part frit. near the column inlet, a little (uneven) void. Sample solvent and mobile phase are incompatible.

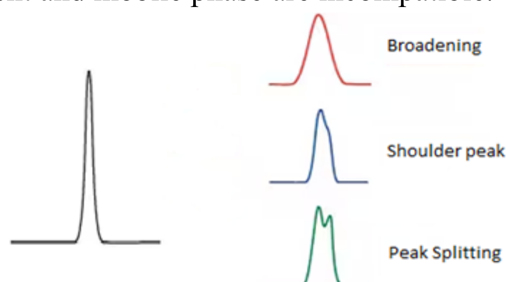

Figure 1: HPLC peaks with some common defects

Peaks Tail on Initial and Later Injections

Several factors, such as how the sample reacts with active sites, incorrect mobile phase pH incorrect kind of column, a little (uneven) void at the column inlet, incorrect injection solvent Damaged column, incorrect sample solvent, secondary interactions, mass and volume overloads, and other extra-column effects including sampling rate and time constant.

Tailing Peaks

By cleaning the column with isopropyl alcohol, we can solve this issue. Typically, column deterioration or input pollution is what causes tailing peaks. The likelihood of tailing peaks will be significantly reduced by carefully maintained columns and guards. Operate at a lower pH, use a highly deactivated column, take into account the likelihood of mass overload, take into account the possibility of column bed deformation, work at a high pH when assessing basic chemicals, and employ a sample clean-up technique as additional reasons for tailing.

Your peaks may start to front or tail, which is a tiny incline at the front or rear, rather than rising and falling in straight lines. This often indicates that the guard or analytical column may be overburdened or worn out. While fronting may be the consequence of issues with the sample solvent, tailing may be caused by a polluted or degraded mobile phase or by interfering mobile components in the sample.

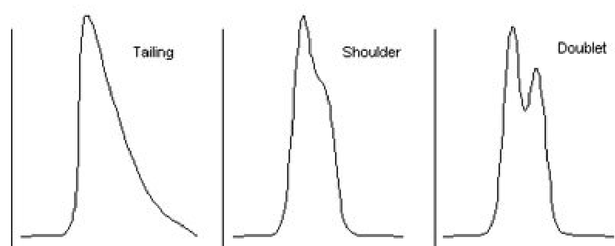

Figure 2: HPLC spectra showing peak tailing, shoulder and doublet peak.

If you have tailing peaks, try analysis first by deleting the guard column; if it fails, replace it. The analytical column could also need to be replaced or restored. Make sure to verify the composition of the mobile phase and the effectiveness of the columns. Contaminated or worn-out analytical column or guard. Contaminated/Deteriorated Mobile Phase.

Contaminated In-Line Filter, Contaminated Guard Column, Replace Guard Column/Insert, Fronting Peaks, Interfering Components In Sample,

Partially Plugged Column Inlet Filter, Remove End-Fitting [1-9]

Column: Relationship, Replace the guard/fritter column. Replace or regenerate the column if it is overloaded. Sample solvent and mobile phase are incompatible. Another sample component might be a shoulder or a slow rise in the baseline prior to the major peak.

Negative peaks:

The main reason why negative peaks occur is because the sample solvent, sample, and mobile phase have different refractive indices. They also result from improper system reconfiguration following standard maintenance.

Rounded Peaks:

running a detector outside of its linear dynamic range. Low recorder gain setting. column is too full. interaction between samples and columns. The time constants for the detector and/or recorder are set too high.

Baseline Drift:

temperature swings within columns. (Even little adjustments result in cyclical baseline rise and decline. primarily impacts conductivity and refractive index detectors, as well as UV detectors with high sensitivity or in indirect photometric mode.) Mobile phase with nonhomogeneity (Drift usually to higher absorbance, rather than cyclic pattern from temperature fluctuation.) accumulation of air or contaminants in the detection cell. plugging in the outlet after the detector. (High pressure cracks cell window, producing noisy baseline.) Changes in flow rate or an issue with mobile phase mixing. In particular while changing mobile phase, slow column equilibration. Contaminated, degraded, or not made with high-quality chemicals is the mobile phase. Strongly held components in the sample might elute as extremely broad peaks, giving the impression that the baseline is rising. (Gradient analyses can aggravate problem.) UV detector is tuned at curve slope rather than absorbance maximum. You might try the next steps.

Eliminate all fluid flow by shutting off the instrument pump.

1. For 5 to 10 minutes, track the baseline. Keep track of any visual changes from the baseline. If so, the issue is with the instrument's fluid flow. If not, there may be an electrical issue or a detector issue.
2. Cut the electrical connections between the detector and the data handling equipment, such as the integrator, PC, and chart recorder (A/D interface). Connect a jump source to the data-handling device's input terminals. (a crocodile clip, paper clip). If the noise persists, the data handling device is the source of the issue [9].

Too low signal-to-noise:

1) An unsuitable fluorescence detector setup might be the cause. The optimal excitation and emission wavelengths should be searched for, the photomultiplier's gain should be optimized, only high-quality mobile phase should be used, and an appropriate reaction time should be established.
2) Potential Root Cause: Improper UV detector settings Solution: Use the operating handbook to adjust slit widths and bandwidths, select a reasonable reaction time, then scan for the optimal absorption wavelength(s).

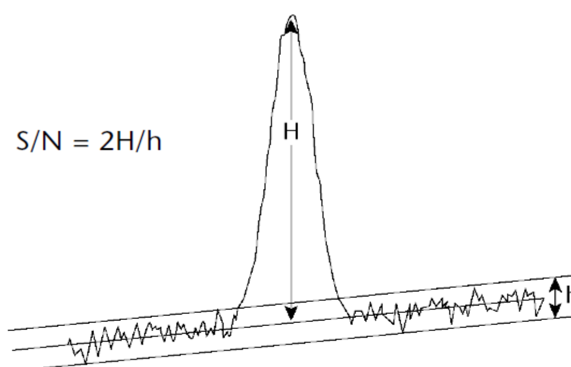

Figure 3: Signal-to-Noise ratio in HPLC spectra

Bubbles Problem in Solvent line:

Consider an in-line degassing accessory unit for your HPLC system as the best solution. Some contemporary HPLC systems come with an integrated degasser.

Alternatives include helium sparging but take care not to change the composition of the mobile phase by allowing volatile components to evaporate. Short-lived degassing is achieved via offline vacuum degassing in an ultrasonic bath, an imperfect approach.

Degas:

It strengthens erratic and noisy baselines. Helium purging, vacuum degassing, and sonication are often employed degassing techniques for HPLC mobile phases [17].

Although boiling is the most efficient method for totally eliminating dissolved air, it is never recommended since it results in the loss of volatile components along with the gases and takes a long time to get the mobile phase up to the necessary ambient temperature conditions. Up to 80% of the dissolved air is removed during helium purging. It is sufficient to use an identical volume of helium for purging the organic-aqueous mobile phase. After some time, the rate of helium delivery may be lowered due to the loss of more volatile mobile phase components caused by excessive purging. During vacuum degassing, more than 60% of dissolved air is removed. Applying vacuum while the mobile phase is being filtered through a 0.45- or 0.22-m porosity membrane filter is one method. On most commercially available systems, on-line vacuum degassing is an option. In a vacuum chamber within the HPLC, porous polymer tubing is used to transport the mobile phase. Gases can escape through the walls of the tube due to its porosity, while liquid remains within. In most laboratories, sonication using ultrasonic baths is a regular practice. However, as sonication alone can only remove up to 30% of dissolved air, it is advised to combine it with any other approach. In low- and high-pressure mixing systems, it is practicable to degas the mobile phase. By combining many different strategies, the majority of the issues related to bubble formation may be resolved. Since most commercially available systems enable online vacuum degassing, sonication in conjunction with online degassing produces good results.

Improper Prime of System:

Instabilities of all types will frequently come from failing to flush all of the lines with newly degassed mobile phase before usage (every day) until all of the old gas loaded mobile phase has been flushed from the system. This might need many liquid column volumes [22–24].

HPLC TROUBLESHOOTING FILTRATION

Even when you have carefully selected an appropriate technique and apparatus for HPLC, issues might still arise, making troubleshooting a regrettable waste of time. A common important factor in troubleshooting is sample preparation. What are some options that you may think about for your sample preparation filtration? Why filtering is advised: Filtering your sample is strongly advised as a minimum preparatory step, even if certain high throughput techniques might not permit it. Unfiltered particles in samples can frequently clog HPLC columns and instrument interfaces, increasing backpressure or stopping the device entirely. The removal of particulate material from samples using filters is a quick, affordable technique to lengthen the life of the instrument and column, increase the rate of analysis done correctly on the first try, and maybe produce superior chromatographic findings. The physical removal of particle species from solutions is often accomplished by filtration. In most cases, the filtering does not impose any chemical separation, thus if sample cleanup is necessary, you might need to think about using other, more selective, sample preparation techniques. One significant exception is ultra filtration, which

forces separation through a semi-permeable membrane using pressure or concentration gradients. Fundamentally, filtering is a quick and practical way to enhance the quality of your analytical results. How then do you choose the proper syringe filter, taking into account factors like extractable, non-specific binding, filter clogging, and filter size [25, 9]?

Purge HPLC:

Purging an HPLC system simply involves running the fresh mobile phase through all the lines to replace any solvent that may still be present from an earlier analysis or wash [18]. As one might anticipate, the volume of tubing in the system affects how much fresh solvent is needed. The on-line vacuum degasser, if equipped, often contributes the most to the amount of tubing. These modules' design needs a considerable volume in order for them to function properly. A standard Agilent 1200 degasser, for instance, has a capacity of around 12 mL for each line.

For the 1200 systems, Agilent advises at least a 30mL complete purge. Therefore, to get a rough idea of how much volume is needed for a purge, double the volume specification of the input tubing, degasser, and pump that you are employing. You could discover that the manufacturer of your specific device has a suggested dosage. Typical flow rates for conventional HPLC systems are 5 mL/min.

Vial Related Issue:

To reduce volatiles' evaporative loss, use Self Sealing Septum. Deciding to choose the incorrect septum might lead to:

- Evaporative loss of sample
- Lack of reproducibility for repetitive injections
- Septum coring
- Needle damage
- Septum dislodging

Peak area grows following the initial injection from the same vial. (first injection – low, latter injections OK). Possible cause: The septum/cap closing around the injection needle results in inadequate venting when the needle pierces it for the FIRST TIME (vacuum creation). Overfilling the vial might make the vacuum scenario where some sample is drawn back out of the needle worse. (Never fill the vial all the way to the top)

Remove the cap and septum from the vial, give many injections, and measure the peak area to see if the septum/cap is the problem.

Symptom: The peak area changes from injection to injection from the same vial (increases/decreases).

Possible reason: Needle-caused septal piercing If a bottom draw port needle is being used, the draw port may be sealed up with septum material.

Check the septum material in the needle draw port and remove or replace it.

Solution: If PTFE/silicone septum is self-sealing: Use a pre-slit PTFE/silicone septum instead. Pre-slit septum will prevent coring and provide effective sealing. Alternately, use PTFE septum. Coring will be stopped by PTFE. But it won't seal again [1–9]

HPLC columns require relatively little care:

However, they can be damaged if:

1. Dropped on the ground
2. Banged around (drawer)
3. Stored in the freezer/ refrigerator (depends on type of solvent)

Column Regeneration:

With 0.1N HCL, the column may be renewed.

Always follow the regeneration instructions from the column vendor. If an issue involves substances that are kept under method circumstances and alter chromatography, regeneration can restore a column's performance. Getting rid of them with more powerful solvents can restore performance. Performance might not be recovered if the surface has undergone chemical alteration, such as hydrolysis of ligands and end capping.

Column Storage:

Keep in the mobile phase for brief periods (less than 72 hours). Keep in shipping solvent for extended periods. The manufacturer's recommended solvent should be used to store the column. Use an organic solvent for bound stages. (eg. MeOH or ACN) -- Hydrolysis is reduced when non-aqueous solvents are used. Polar organic mobile phases cause some bonded phases (CN) to become unstable. Then, storage in buffer or water is acceptable. columns that can be kept in buffered solvents or aqueous SEC packings, such as water, or in ion exchangers. However, employing 0.05% sodium azide in the mobile phase or a little amount of an organic solvent (acetonitrile 5% or methanol 10%) can prevent the development of microorganisms.

Aggressive buffers that should be avoided:

In the alkaline pH range, phosphate has been reported to be more aggressive than other buffers. Therefore, I do not advise using alkaline phosphate buffers that are packaged in inorganic-organic hybrid or silica-based materials. The pH range of phosphate is somewhat constrained. Although organic amine may easily replace ammonia, which is also extremely harsh.

Colum damage by Air:

You can pull air into your HPLC pump if the reservoir gets dry, but the pump won't push air through the system. Liquid, not air, is what HPLC pumps are made to move. They lose their prime and stop pumping when they are filled with air [26]. Before the pump completely stops, you might pump in a few bubbles of air, but if you leave the pump going all night, it won't have continuously pumped air through the column. It is improbable that it would have led to issues even if it had. You must first re-prime the pump by removing all of the air from the pipes in order to fix the issue. I'd take off the column before beginning. Start by completely degassing the solvent. This could be adequate if you utilize an in-line degasser, but if not, I advise helium sparging. After that, release the purge valve located at the pump output and prime the pump as usual. For some pumps, this entails using a syringe to inject fluid into the tubing that connects the reservoir(s) and pump [30].

Gradient Dwell volume:

Between the gradient's mixing point and the column intake, there is volume. This volume is also known as a gradient delay volume since it postpones the gradient's beginning.

MOBILE PHASE pH and pH BUFFERS

One of the most crucial choices you make in deciding the outcome of your HPLC investigation is the mobile phase you will employ. The mobile phase type, together with its polarity and quality, has a significant impact on the effectiveness and accuracy of your findings, second only to the column. Anyone utilizing an HPLC, whether directly or as part of a manufacturing/release process, should be aware of what mobile phase is and how it functions [20, 21] for something this crucial. Mobile phase is used to transport the sample through the column while slowing down and separating the sample's constituent parts. These elements are segregated in the column according to their size, shape, charge, polarity, hydrophobic state, and binding ability. The sample "slows down" in comparison to the mobile phase, which triggers the separation. Individual components that start to separate from the mobile phase are then recognized and measured. In addition to influencing retention and selectivity, pH also regulates a method's accuracy and precision [27, 28]. Since the parameters of the sample and the intended analytes affect which pH is optimum for a run, there is no one "best" pH for mobile phase. The requirement to have a constant pH that stays at the specified level throughout the mobile phase's useable life is universal. This can take anything from a few minutes or hours (the time it takes to cycle a sample set through the HPLC) to as long as six months or more in certain closed systems. When a sample could have an

impact on the pH during a normal run, buffer should be used to create the mobile phase [29]

- pH Effects Ionization
- Silica Surface of Column
- Sample Components of Interest
- Buffers
- Resist Changes in pH and Maintain Retention
- Improve Peak Shape for Ionizable Compounds
- Effects Column Life
- Low pH strips Bonded Phase
- High pH Dissolves Silica
- Column and HPLC Cleaning:

Frequency: [19]

- General cleaning Daily
- Syringe, loop & injector plunger seal: After completion of each analysis.
- Flow cell, pipeline, suction filter: When Problem Arise
- Keep the record of cleaning.

Flush with stronger solvent then your mobile phase.

A. Water Soluble Samples Flush with the following:

1. Flush with warm (50 °C) distilled water
2. Acetonitrile
3. Methanol

B. Samples Not Soluble in Water Flush with the following:

1. Methylene chloride
2. Hexane
3. Isopropyl Alcohol (Also Improve Tailing Factor)

Always remember to wash your system and column with acetonitrile after a run: Water (50%:50%) or Water: Methanol (50%:50%) Using the same solvent, clean the area by removing the column and replacing it with a union. [30, 31]

CONCLUSION

It argues that while dealing with HPLC issues, prevention is always preferable to treatment. By the way, these concerns are frequently unrelated to the column and might be brought on by chemistry and instrument problems, such as pH of the mobile phase, instrument connections, detector settings, and metal contamination. Start by asking the right questions, then look for the answers; they will point you in the direction of solutions. Research, product evaluation, and environmental monitoring are among applications for HPLC. The maintenance of the HPLC system is the greatest way to prevent HPLC issues.

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